Characterization of North American *Armillaria* species: genetic relationships determined by ribosomal DNA sequences and AFLP markers

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Summary

Phylogenetic and genetic relationships among 10 North American *Armillaria* species were analysed using sequence data from ribosomal DNA (rDNA), including intergenic spacer (IGS-1), internal transcribed spacers with associated 5.8S (ITS + 5.8S), and nuclear large subunit rDNA (nLSU), and amplified fragment length polymorphism (AFLP) markers. Based on rDNA sequence data, the nLSU region is less variable among *Armillaria* species than the ITS + 5.8S and IGS-1 regions (nLSU < ITS + 5.8S < IGS-1). Phylogenetic analyses of the rDNA sequences suggested *Armillaria mellea*, *A. tabescens* and *A. nabsnona* are well separated from the remaining *Armillaria* species (*A. ostoyae*, *A. gemina*, *A. calvescens*, *A. sinapina*, *A. gallica*, NABS X and *A. cepistipes*). Several *Armillaria* species (*A. calvescens*, *A. sinapina*, *A. gallica*, NABS X and *A. cepistipes*) clustered together based on rDNA sequencing data. Based on the isolates used in this study, it appears that techniques based on IGS-1, ITS + 5.8S, and/or D-domain/3′ ends of nLSU are not reliable for distinguishing *A. calvescens*, *A. sinapina*, *A. gallica* and *A. cepistipes*. However, AFLP data provided delineation among these species, and AFLP analysis supported taxonomic classification established by conventional methods (morphology and interfertility tests). Our results indicate that AFLP genetic markers offer potential for distinguishing currently recognized North American Biological Species (NABS) of *Armillaria* in future biological, ecological and taxonomic studies.

1 Introduction

Armillaria is a genus that comprises over 30 species of wood-decaying fungi. These species are primarily recognized for their association with root and butt rot of woody plants, but they are also important decomposers within many forested environments (e.g. natural, urban and agroforestry). In addition, *Armillaria* species are ecologically diverse, with variable pathogenicity, host specificity and other environmental requirements. For example, *Armillaria ostoyae* (Romagn.) Herink is a primarily aggressive pathogen on conifers, *A. mellea* (Vahl: Fr.) Kummer is a primarily aggressive pathogen on hardwoods, and *A. gallica* Marxmüller & Romagn. is predominantly saprophytic (Shaw and Kile 1991).

Based on morphology and *in vitro* compatibility of isolates, *Armillaria* species in North America were initially grouped into 10 North American Biological Species (NABS) (Hintikka 1973; Korhonen 1978; Anderson and Ullrich 1979; Motta and Korhonen 1986; Termorshuizen and Arnolds 1987, 1997; Bérubé and Dessureault 1988; Banik et al. 1996; Volk et al. 1996). The absence of an annulus separates *A. tabescens* (Scop.) Emel from other North American species of *Armillaria* (*A. ostoyae*, *A. gemina* Bérubé & Dessur., *A. calvescens* Bérubé & Dessur., *A. sinapina* Bérubé & Dessur., *A. mellea*, *A. gallica*, *A. nabsnona* Volk & Burdsall, NABS X and *A. cepistipes* Velen.). In addition to mating tests, the other *Armillaria* species, except NABS X, have been formally described on a basis of macro-/microscopic and distributional characters (Volk and Burdsall 1995). However,

basidiospores are unavailable for most isolates of *Armillaria* species, and it can be impractical or unreliable to identify species using other characteristics. Basidioma morphology has also been used to identify *Armillaria* species (Motta and Korhonen 1986; Termorshuizen and Arnolds 1987, 1997; Bérubé and Dessureault 1988), but basidioma production can be unpredictable or rare.

Several molecular methods were developed to augment identification of *Armillaria* species and determine phylogenetic relationships among *Armillaria* species (Anderson et al. 1987, 1989; Jahnke et al. 1987; Smith and Anderson 1989; Smith et al. 1990; Anderson and Stasovski 1992; Miller et al. 1994; Harrington and Wingfield 1995; Schulze et al. 1995, 1997; Chillali et al. 1998; Piercey-Normore et al. 1998; Terashima et al. 1998; White et al. 1998; Coetzee et al. 2000, 2001, 2003; Kim et al. 2000; Otieno et al. 2003; Gezahgne et al. 2004; Pérez-Sierra et al. 2004). Anderson and Stasovski (1992) determined the DNA sequences of intergenic spacer (IGS-1) and assessed phylogenetic relationships among the NABS of *Armillaria*. In addition, Piercey-Normore et al. (1998) compared anonymous nucleotide sequences from eight NABS of *Armillaria* to evaluate their phylogeny. Previous molecular phylogenetic studies indicated that *A. calvescens*, *A. sinapina*, *A. gallica*, *A. nabsnona*, NABS X and *A. cepistipes* represent a closely related group that is well separated from *A. ostoyae*, *A. gemina*, *A. mellea* and *A. tabescens* (Anderson and Stasovski 1992; Piercey-Normore et al. 1998). Subsequently, Coetzee et al. (2000, 2001, 2003) addressed the phylogenetic relationships among isolates of *A. mellea* s.s. and other Southern Hemisphere *Armillaria* species based on DNA sequences from the IGS-1 as well as the internal transcribed spacer (ITS) regions.

The 5′ end of the nuclear large subunit (nLSU) rDNA gene (proximal to ITS2), which comprises the divergent domains D1–D3 region (Michot et al. 1984, 1990), has been used to study phylogenetic relationships of agaric fungi (Hopple and Vilgalys 1999; Moncalvo et al. 2000, 2002). This region contains the most phylogenetically informative sites in the nLSU rDNA gene (Kuzoff et al. 1998; Hopple and Vilgalys 1999; Fell et al. 2000; Weiss and Oberwinkler 2001). However, this region has not been applied to the study of phylogenetic relationships among the NABS of *Armillaria*.

The objectives of this study were to: (i) determine phylogenetic relationships among 10 NABS of *Armillaria* using rDNA (IGS-1, ITS + 5.8S and nLSU) sequence data and (ii) assess genetic relationships among NABS of *Armillaria*, especially within the closely related group that includes *A. calvescens*, *A. sinapina*, *A. gallica*, *A. nabsnona*, NABS X and *A. cepistipes*, using high-resolution amplified fragment length polymorphism (AFLP) markers. Attaining these objectives will also provide a better characterized set of diploid tester strains (Kim et al. 2000) for use in biological, ecological and taxonomic studies of *Armillaria* species.

2   Materials and methods

2.1   Fungal isolates

Tested species included *A. ostoyae*, *A. gemina*, *A. calvescens*, *A. sinapina*, *A. mellea*, *A. gallica*, *A. nabsnona*, NABS X, *A. cepistipes* and *A. tabescens*. Three isolates representing each of the 10 North American *Armillaria* species were included in the study, most of which were previously characterized for nuclear DNA content (Kim et al. 2000). Isolates originated from basidioma (stipe or context) tissue or mass-spore cultures, and were previously identified by various investigators using haploid × haploid mating or haploid × diploid pairing tests (Table 1). To ensure that each isolate represented a distinct genet (vegetative clone), isolates were selected from different geographical regions or were tested by somatic pairing (Anderson and Kohn 1995; Kim et al. 2000). All isolates used in

Table 1. Armillaria isolates used in phylogenetic analysis

Species	Collection	Isolate[1]	Origin	Source tissue	ITS-1, 5.8S, ITS-2 and partial nLSU	IGS-1 and partial nLSU
					GenBank accession No.	
A. ostoyae	DMR20[3]	ST1	New Hampshire, USA	Multisporous	AY213552	AY509154
	AMM9067[3]	ST2	Washington, USA	Basidioma	AY213553	AY509155, AY509156
	P1404[4]	P1404	Idaho, USA	Basidioma	AY213554	AY509157
A. gemina	JJL153[5]	ST8	New York, USA	Basidioma	AY213555	AY509158, AY509159
	JJW64[5]	ST9	New York, USA	Basidioma	AY213556, AY213557	AY509160, AY509161
	MIELKE[3]	ST11	West Virginia, USA	Unknown	AY213558	AY509162
A. calvescens	JB56A[6]	ST3	Quebec, Canada	Basidioma	AY213559	AY509163
	PR-3[3]	ST17	Michigan, USA	Basidioma	AY213560, AY213561	AY509164
	FFC-7[3]	ST18	Michigan, USA	Basidioma	AY213562	AY509165, AY509166
A. sinapina	SP81-1[7]	M50	British Columbia, Canada	Basidioma	AY213563, AY213564	AY509167
	AMM9065[3]	ST12	Washington, USA	Basidioma	AY213565	AY509168
A. mellea	CF-2[3]	ST13	Michigan, USA	Multisporous	AY213566, AY213567	AY509169, AY509170
	GB934[3]	ST5	Virginia, USA	Multisporous	AY213584, AY213585	AY509185, AY509186
	A3[3]	ST20	Wisconsin, USA	Basidioma	AY213586	AY509187
	TCH-2[3]	ST21	New Hampshire, USA	Multisporous	AY213587	AY509188
A. gallica	SP81-29[7]	M70	British Columbia, Canada	Basidioma	AY213568	AY509171
	EL-1[3]	ST22	Michigan, USA	Basidioma	AY213569, AY213570	AY509172
	MA-1[3]	ST23	Wisconsin, USA	Basidioma	AY213571	AY509173
A. nabsnona	C21[4]	C21	Idaho, USA	Basidioma	AY213572	AY509174, AY509175
	M90[7]	M90	British Columbia, Canada	Basidioma	AY213573	AY509176, AY509177
NABS[2] X	SHAW,C.C[3]	ST16	Alaska, USA	Multisporous	AY213574	AY509178
	837[4]	837	Idaho, USA	Basidioma	AY213575, AY213576	AY509179
	D82[4]	D82	Idaho, USA	Basidioma	AY213577, AY213578	AY509180
	POR100[4]	POR100	Idaho, USA	Basidioma	AY213579, AY213580	AY509181
A. cepistipes	SP82-14[7]	M110	British Columbia, Canada	Basidioma	AY213581	AY509182
	SP83-07[7]	S20	British Columbia, Canada	Basidioma	AY213582	AY509183
	HH14867[3]	W113	Washington, USA	Basidioma	AY213583	AY509184
A. tabescens	AT-MU-S2[8]	AT-MU-S2	South Carolina, USA	Stipe	AY213588	AY509189, AY509190
	OOI-99[8]	OOI-99	Georgia, USA	Basidioma	AY213589	AY509192
	OOI-210[8]	OOI-210	Georgia, USA	Basidioma	AY213590	AY509191

[1] Identification code in this paper.
[2] NABS = North American Biological Species.
[3] Identified by the USDA Forest Service Center for Forest Mycology Research, Madison, WI, USA.
[4] Identified by G. I. McDonald.
[5] Identified by J. Worrall.
[6] Identified by J. Bérubé.
[7] Identified by D. Morrison.
[8] Identified by G. Schnabel.

this study are maintained as archival cultures at the USDA Forest Service, Forestry Sciences Laboratory in Moscow, ID, USA.

2.2   DNA sequencing

For use in DNA sequence analysis, isolates were maintained in Petri dishes on 3% malt-agar medium (3% malt extract, 1.5% peptone, 3% glucose, 1.5% agar) and incubated at 22°C in the dark. The IGS-1, ITS + 5.8S and 5′ (divergent domains D1, D2 and D3; MICHOT et al. 1984, 1990) + 3′ (ca 207 nucleotides upstream from IGS-1) ends of nLSU rDNA regions were amplified using polymerase chain reaction (PCR). Template DNA was derived from scrapings of actively growing mycelial cultures (1–2 weeks old). Primers LR12R/O-1 (VELDMAN et al. 1981; DUCHESNE and ANDERSON 1990), ITS-1F/ITS-4 (WHITE et al. 1990; GARDES and BRUNS 1993) and 5.8SR/LR7 (MONCALVO et al. 2000) were used to amplify the 3′ nLSU + IGS-1, ITS + 5.8S rDNA, and nLSU D-domains, respectively (Fig. 1). Each 50-μl reaction mixture contained template DNA obtained from scraping cultured mycelia (or no DNA template for negative control), 2.5 U *Taq* polymerase (Applied Biosystems, Inc., Foster City, CA, USA), PCR reaction buffer (supplied with *Taq* enzyme), 4 mM MgCl$_2$, 200 μM dNTPs, and 0.5 μM of each primer. The PCR conditions were as follows: (i) for 3′ nLSU + IGS-1, 95°C for 1 min 30 s, 35 cycles of 95°C for 1 min 30 s, 60°C for 40 s, and 72°C for 2 min, and finally 72°C for 10 min; (ii) for ITS + 5.8S, 94°C for 2 min 30 s, 35 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min 30 s, and finally 72°C for 10 min; and (iii) for nLSU-D domains, 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min, and finally 72°C for 5 min. All PCRs were conducted by MJ PTC-200 thermocycler (Bio-Rad Laboratories, Waltham, MA, USA). Intact PCR products were electrophoresed in 1.5% agarose gels using 0.5X TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA) buffer. Gels were stained with ethidium bromide (0.5 μg/ml) and bands were visualized using UV light. PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH, USA), then sequenced with an ABI 377 or ABI 3700 DNA sequencer at the Davis Sequencing Facility (Davis, CA, USA). IGS-1 and ITS + 5.8S regions were sequenced with the same primers used for initial amplification, while the nLSU-D domains were sequenced using the LR0R, LR15 and LR5 primers (Fig. 1) (MONCALVO et al. 2000).

2.3   Sequencing editing and alignment

Sequences were edited and aligned manually with BIOEDIT software (HALL 1999). Sequence editing was performed independently by two separate researchers to minimize errors. An additional precaution was to sequence rDNA regions in both directions and duplicate all steps (from PCR to DNA sequencing) for all of the *Armillaria* isolates we tested. Careful attention was paid to sequences containing heterogeneous products. Polymorphisms were coded with the IUPAC codes for ambiguous nucleotides. These polymorphisms were not regarded as ambiguous but rather the result of heterogeneous products because of variation within rDNA repeat units (CREASE and LYNCH 1991). In this study, heterogeneity was detected when a chromatogram contained either single nucleotide polymorphisms
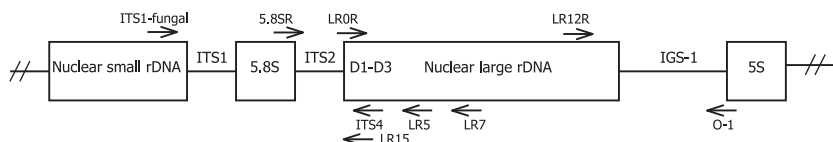

Fig. 1. Diagram of rDNA repeat. The arrows correspond to the annealing sites and direction for several primers

(represented by a double peak occurring at a single nucleotide position) or a 'frame-shift' (overlapping peaks due to length variation among the rDNA repeat units). When possible, heterogeneous sequences were split into homogeneous sequence representations by one of the three methods described in HANNA (2005) before phylogenetic analyses. The different sequence types from an individual isolate were given a letter code (e.g. A or B) after genet names (Figs 2 and 3; Table 1). For example, *A. gemina* isolate ST9 has two IGS-1 sequence types, ST9-A and ST9-B, respectively, whereas *A. gemina* isolate ST11 produced a homogenous sequence in the IGS-1 region, and has only one sequence type. All sequences used in phylogenetic analyses have been deposited into GenBank (AY213552–AY213590; AY509154–AY509192) (Table 1).

2.4 Phylogenetic analyses of IGS-1, ITS + 5.8S and nLSU sequences

Phylogenetic analyses were performed for each of three rDNA regions (IGS-1, ITS + 5.8S and 5′/3′ ends of nLSU) using neighbor-joining (NJ) (SAITOU and NEI 1987), Parsimony and Bayesian inference methods. Most of the genets of this study contained unique sequence types; however, any duplicate sequence types were removed from the dataset before phylogenetic analyses.

Any gaps in sequence alignments were treated as missing and coded using a simple gap-coding method (SIMMONS and OCHOTERENA 2000). NJ analysis was performed using PAUP* (4.0b10) (SWOFFORD 2001) with 1000 bootstrap replicates (FELSENSTEIN 1985). Parsimony analysis was performed using PAUP* (4.0b10). The bootstrap method (FELSENSTEIN 1985) with heuristic search was used with 1000 bootstrap replicates on each dataset to obtain 50% bootstrap majority-rule consensus trees. Multistate taxa were interpreted as polymorphisms, starting trees were obtained via stepwise addition with random addition sequence of 10 replicates, tree-bisection-reconnection was used for keeping one tree at each step, and the steepest descent option was not in effect. The analysis of the nLSU was performed with MaxTrees set to auto increase, while the IGS-1 and ITS + 5.8S regions were set to a maximum of 10 000 trees because of computational runtime limitations.

Bayesian analysis was performed by MRBAYES v3.0B4 (HUELSENBECK and RONQUIST 2001). Bayesian inference of phylogeny calculates the posterior probability of phylogenetic trees. To select appropriate evolutionary models to use in Bayesian analysis, MRMODEL-TEST 1.0b (NYLANDER 2003) was used on each data set. For the nLSU and ITS + 5.8S data sets, Hasegawa-Kishino-Yano (HKY) model (HASEGAWA et al. 1985) was selected. And for the IGS-1 data set, HKY + G (Γ; YANG 1993) model was selected. Four chains were run for 3×10^6 generations generating files with 30 001 trees, the first 6000 of these trees were discarded as the 'burn-in' of the chains. The remaining 24 001 trees were used to make 95% majority consensus trees using PAUP* (4.0b10).

Ambiguous alignment of sequences due to overlapping indels of different lengths may lead to misleading phylogenies (HALL 2001); therefore, we used a conservative approach (ERIKSSON et al. 2003) and refrained from comparing sequences that resulted in ambiguous alignment. In the IGS-1 and ITS + 5.8S regions, *A. mellea* and *A. tabescens* would not align with the other species without ambiguity. Preliminary analysis suggested that the phylogenetic placement of *A. mellea* and *A. tabescens* would be significantly resolved using the nLSU D-domains and 3′ ends of nLSU; therefore, these species were excluded from the IGS-1 and ITS + 5.8S datasets to remove ambiguity and give greater resolution to the comparison of the more closely related individuals/species. *Armillaria ostoyae* and *A. gemina* were used as outgroups for the IGS-1 and ITS + 5.8S regions, and *A. mellea* was used as an outgroup for the nLSU region.

All phylogenetic trees generated from rDNA sequence data have been deposited into TreeBASE (study accession number = S1452).

2.5 AFLP markers

To generate biological material for AFLP studies, isolates were cultured on 0.2-μm pore, nylon filters (Millipore Corp., Billerica, MA, USA) that overlayed the 3% malt-agar medium in Petri dishes, then incubated at 22°C in the dark for 2 weeks. Approximately 100 mg fresh mycelia was scraped from the nylon filter and used for DNA extraction. DNeasy Plant Mini DNA extraction kits (Qiagen Inc., Valencia, CA, USA) were used to extract and purify all DNA samples following the protocol of the manufacturer. DNA was quantified for AFLP analysis using a TD-360 fluorometer (Turner Designs, Sunnyvale, CA, USA).

Amplified fragment length polymorphism analyses were performed following the protocol of Vos et al. (1995). For restriction digests, 350 ng of genomic DNA was digested with *Eco*RI and *Mse*I (New England BioLabs, Inc., Beverly, MA, USA) to serve as the template. Resulting DNA fragments were ligated to adapters and diluted 1 : 10 with sterile distilled water prior to pre-amplification. Pre-amplification reaction mixtures (total 30 μl) contained 6 μl of diluted restriction/ligation mixture as template, 10X PCR buffer (Applied Biosystems, Inc.), 3 mM $MgCl_2$, 200 μM dNTPs, 300 nM of each pre-selective primer (*Eco*RI-no extension and *Mse*I-C), and 1.5 U Ampli*Taq*® DNA polymerase (Applied Biosystems, Inc.). The PCR conditions for pre-amplification were as follows: 72°C for 2 min, 20 cycles of 94°C for 40 s, 56°C for 1 min, and 72°C for 3 min, and finally 72°C for 10 min.

For the selective amplification, we screened 24 different primer combinations using DNA from 10 isolates of *Armillaria* species. Among several promising selective primer combinations, we choose three (*Eco*RI-AA/*Mse*I-CTG, *Eco*RI-AG/*Mse*I-CAC, and *Eco*RI-AG/*Mse*I-CTT) that produced the most manageable band number and interpretable banding pattern. Selective amplification mixtures (total 25 μl) contained 5 μl of diluted pre-amplification products [1 : 20 with low TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)] as a template, 10X PCR buffer (Applied Biosystems, Inc.), 2.4 mM $MgCl_2$, 300 μM dNTPs, 100 nM of *Eco*RI-AA or -AG primers, 300 nM of *Mse*I-CTG, -CAC, or -CTT primers, and 1.25 units of Ampli*Taq*® Gold polymerase (Applied Biosystems, Inc.). Amplifications were performed using the following PCR conditions: 94°C for 2 min, 14 cycles of 94°C for 30 s (1°C per second to 65°C), 65°C for 30 s (−0.7°C per cycle), and 72°C for 2 min, 20 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, and finally 72°C for 10°min. All PCR were conducted using a MJ PTC-200 thermocycler (Bio-Rad Laboratories).

Selective amplification products were separated in an ABI 3700 DNA automated sequencer (Applied Biosystems, Inc.) at the University of Wisconsin – Biotechnology Center (Madison, WI, USA). GENOTYPER 3.7 NT (Applied Biosystems, Inc.) was used to identify peaks with a fluorescent intensity greater than the threshold value (ca 300 units) in at least one sample. Categories were made from these identified peaks for the scoring of all samples.

2.6 AFLP data analysis

The AFLP bands were scored as present or absent using GENESCAN 3.7 NT (Applied BioSystems, Inc.) and a binary matrix was developed with molecular sizes ranging from 71 to 616 bp. The resulting binary matrix was used to calculate genetic distances (NEI and LI 1979) among *Armillaria* genets and distances were graphically displayed by unweighted paired-group method with arithmetic means (UPGMA) using PAUP* (4.0b10). The UPGMA dendrograms were constructed with confidence estimates assigned to its topology based on 1000 bootstrap replicates.

The UPGMA tree generated from AFLP marker data has been deposited into TreeBASE (study accession number = S1452).

3 Results

3.1 Sequence data from IGS-1, ITS + 5.8S and nLSU rDNA regions

The IGS-1 data set consisted of 29 unique sequences that each contained 606 total characters, of which 147 (24%) varied and 85 (14%) were parsimony informative. An optimum sample tree from the heuristic search (limited to 10 000 trees) yielded a total length of 526 steps with consistency index (CI) of 0.992, a retention index (RI) of 0.992, and a rescaled consistency index (RC) of 0.984. The resulting 50% majority-rule bootstrap-consensus tree from the parsimony analysis and the 95% majority-rule consensus tree (based on posterior probability) from the Bayesian analysis showed nearly identical topologies (Fig. 2).

The ITS + 5.8S data set consisted of 26 unique sequences that each contained 792 total characters, of which 55 (6.9%) varied and 19 (2.4%) were parsimony informative. An optimum sample tree from the heuristic search (limited to 10 000 trees) yielded a total length of 93 steps (CI = 0.968, RI = 0.952 and RC = 0.921). The resulting 50% majority-rule bootstrap-consensus tree from the parsimony analysis and the 95% majority-rule consensus tree (based on posterior probability) from the Bayesian analysis showed identical topologies (Fig. 3).

The nLSU D-domains and 3′ ends data set consisted of 22 unique sequences that each contained 1197 total characters, of which 82 (6.9%) varied and 42 (3.5%) were parsimony informative. With optimality criterion set to parsimony, 198 equally parsimonious trees were revealed. An optimum sample tree from the heuristic search yielded a total length of 1436 steps with CI = 0.999, RI = 0.979 and RC = 0.978. The resulting 50% majority-rule bootstrap-consensus tree from the parsimony analysis and the 95% majority-rule consensus tree (based on posterior probability) from the Bayesian analysis showed a high degree of congruence (Fig. 4).

3.2 Phylogenetic analyses from rDNA sequence data

Bayesian, Parsimony and NJ (data not shown) analyses showed remarkable congruence for all three regions analysed, as exemplified by the Bayesian and Parsimony trees shown in Figs 2–4.

Phylogenetic analyses of the IGS-1 region revealed two major clades: (i) *A. nabsnona* (posterior probability 100%, bootstrap support 100%), and (ii) the other *Armillaria* species that were analysed, NABS X, *A. gallica*, *A. calvescens*, *A. sinapina* and *A. cepistipes* (100%, 100%). Within the second clade, a cluster of NABS X species appears separate (96%, 68%) (Fig. 2). In general, *A. ostoyae* and *A. gemina* were well separated from each other as outgroups. One genet (ST9) of *A. gemina* possessed two IGS-1 sequence types (ST9-A and ST9-B), one of which clustered with *A. ostoyae* (Fig. 2).

Phylogenetic analyses of the ITS + 5.8S region provided two major clades and several sequence types: (i) *A. nabsnona* and NABS X sequence type A (837-A, D82-A and POR100-A) (96%, 88%), (ii) *A. cepistipes* and one genet of *A. sinapina* (M50) (100%, 68%), and (iii) the remaining unclustered *Armillaria* species/sequences that were analysed, NABS X sequence type B (837-B, D82-B and POR100-B), *A. sinapina*, *A. gallica* and *A. calvescens* (Fig. 3).

Phylogenetic analyses of the nLSU rDNA region demonstrated two major clades: (i) *A. tabescens* (97%, 93%), and (ii) the remaining *Armillaria* species, *A. ostoyae*, *A. gemina*, NABS X, *A. gallica*, *A. calvescens*, *A. sinapina*, *A. nabsnona* and *A. cepistipes* (100%, 89%) (Fig. 4). A separate cluster of *A. ostoyae* (ST2 and P1404) species was apparent (99% posterior probability) with moderate bootstrap support (62%) (Fig. 4). An *A. nabsnona* cluster also appeared separate from other *Armillaria* species with moderate bootstrap support (64%), but no support from Bayesian probability (Fig. 4).
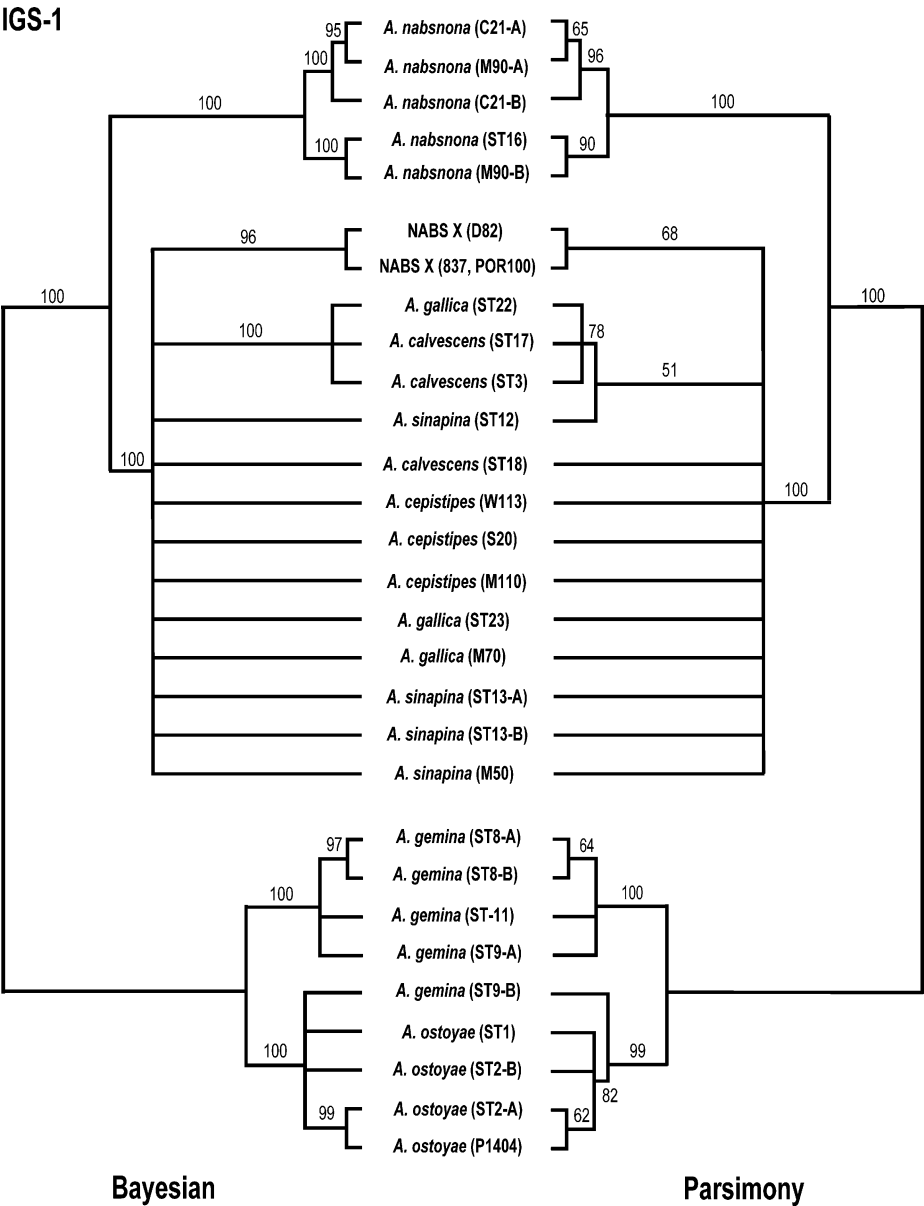
IGS-1

Fig. 2. Bayesian and parsimony trees generated from intergenic spacer (IGS-1) rDNA sequences in *Armillaria* species. To the left, a 95% majority-rule posterior probability-consensus tree based on 24 001 trees from the Bayesian inference analysis. Numbers above branches indicate their respective posterior probabilities. To the right, a 50% majority-rule bootstrap-consensus tree from the parsimony analysis. Bootstrap supports are indicated above branches based on 1000 bootstrap replicates
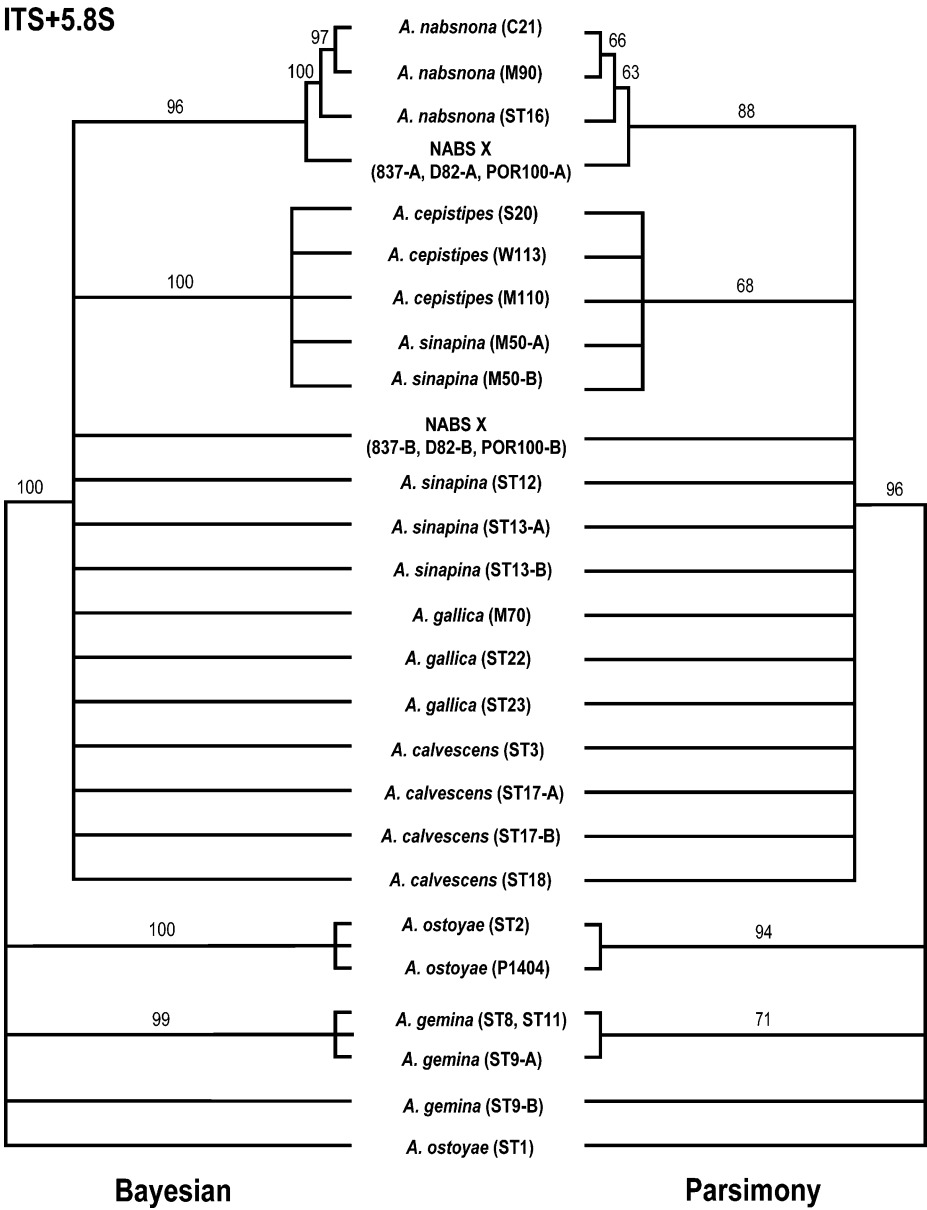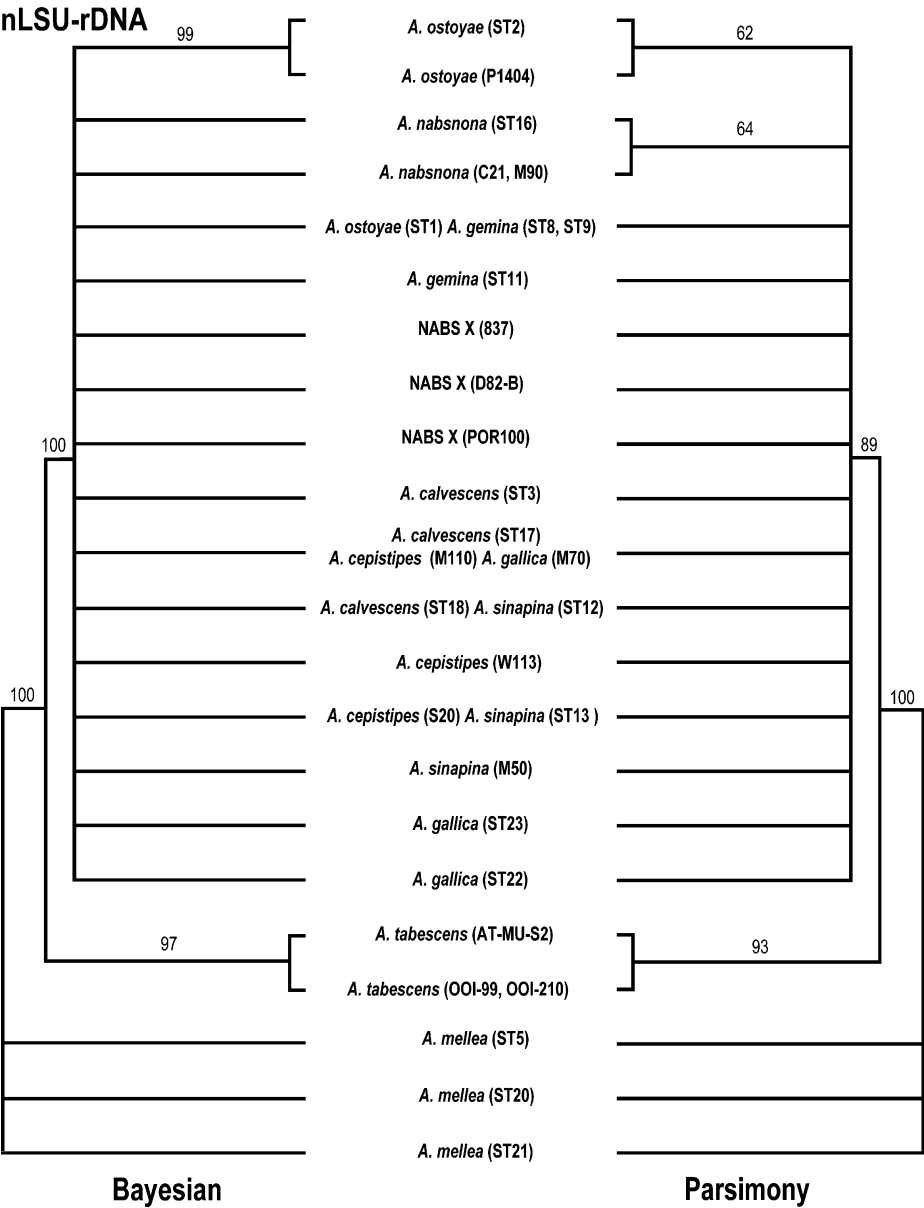
ITS+5.8S

Fig. 3. Bayesian and parsimony trees generated from internal transcribed spacer including 5.8S (ITS + 5.8S) rDNA sequences in *Armillaria* species. To the left, a 95% majority-rule posterior probability-consensus tree based on 24 001 trees from the Bayesian inference analysis. Numbers above branches indicate their respective posterior probabilities. To the right, a 50% majority-rule bootstrap-consensus tree from the parsimony analysis. Bootstrap supports are indicated above branches based on 1000 bootstrap replicates

nLSU-rDNA

Fig. 4. Bayesian and parsimony trees generated from nuclear large subunit rDNA (nLSU) rDNA sequences in *Armillaria* species. To the left, a 95% majority-rule posterior probability-consensus tree based on 24 001 trees from the Bayesian inference analysis. Numbers above branches indicate their respective posterior probabilities. To the right, a 50% majority-rule bootstrap-consensus tree from the parsimony analysis. Bootstrap supports are indicated above branches based on 1000 bootstrap replicates

Table 2. Description of unique amplified fragment length polymorphism (AFLP) markers among North American Armillaria species

Primer combination[1]	Polymorphic loci[2]	A. ost loci[3]	A. gem loci	A. cal loci	A. sin loci	A. mel loci	A. gal loci	A. nab loci	NABS X loci	A. cep loci	A. tab loci
Eaa/Mctg	139	aactg448 aactg575	aactg180 aactg224		aactg398	aactg234 aactg282	aactg240	aactg323 aactg405	aactg276 aactg278	aactg298	aactg153 aactg256 aactg355 aactg475 aactg569
Eag/Mcac	69				agcac226	agcac181 agcac256		agcac269		agcac231	agcac119 agcac130 agcac330
Eag/Mctt	96		agctt167 agctt293 agctt300		agctt298	agctt108		agctt408	agctt282	agctt136 agctt244 agctt362	agctt163 agctt178 agctt193 agctt194 agctt201 agctt267 agctt506
Total	304	2	5		3	5	1	4	3	5	15

A. ost = A. ostoyae; A. gem = A. gemina; A. cal = A. calvescens; A. sin = A. sinapina; A. mel = A. mellea; A. gal = A. gallica; A. nab = A. nabsnona; NABS = North American Biological Species; A. cep = A. cepistipes; A. tab = A. tabescens.
[1] AFLP-selective +2/+3 primer combination – Eaa/Mctg (EcoRI-AA/MseI-CTG), Eag/Mcac(EcoRI-AG/MseI-CAC), Eag/Mcac(EcoRI-AG/MseI-CAC), and Eag/Mctt (EcoRI-AG/MseI-CTT).
[2] The number of polymorphic markers found among all of the Armillaria genets.
[3] Markers that are unique to species.

3.3   AFLP marker data

The three selective primer combinations amplified a total of 307 fragments, of which 304 (99%) were polymorphic. Each of the 30 *Armillaria* genets had a unique AFLP phenotype. However, some AFLP markers appeared to be species specific and were shared by all three isolates of an individual *Armillaria* species, with the exception of *A. calvescens* (Table 2). Of the 304 polymorphic AFLP loci evaluated, two were unique to *A. ostoyae*, five unique to *A. gemina*, three unique to *A. sinapina*, five unique to *A. mellea*, one unique to *A. gallica*, four unique to *A. nabsnona*, three unique to NABS X, five unique to *A. cepistipes*, and 15 unique to *A. tabescens* (Table 2). For example, loci aactg448 (selective primer combination *Eco*RI-AA/*Mse*I-CTG) and aactg575 were only found in the three genets of *A. ostoyae*.

3.4   Genetic analysis of AFLP marker data

A cluster analysis disclosed three major clades: *A. tabescens* (bootstrap support 100%), *A. mellea* (100%), and the remaining North American *Armillaria* species (81%) (Fig. 5). The third clade can be further separated into two groups: (i) *A. ostoyae* and *A. gemina* (83%), and (ii) *A. sinapina*, *A. cepistipes*, NABS X, *A. nabsnona*, *A. calvescens* and *A. gallica* (98%) (Fig. 5). Within the second major group, *A. sinapina* and *A. cepistipes* were contained within a cluster (81%) that was separate from other *Armillaria* species (NABS X, *A. nabsnona*, *A. calvescens* and *A. gallica*) (Fig. 5). Overall, genetic analysis of AFLP marker data indicated strong support for intraspecific clustering (Fig. 5).

4   Discussion

Species of *Armillaria* in North America can be placed into three to four major groups based on morphological, biological and molecular evidence (Anderson and Stasovski 1992; Korhonen 1995; Piercey-Normore et al. 1998). Korhonen (1995) divided the Northern Hemisphere *Armillaria* species into four clusters on the basis of morphological and biological features. He designated *A. mellea* as a single species cluster and *A. tabescens* and *A. monadelpha* (Morgan) Guillaumin & Mohammed as a two-species cluster. *Armillaria borealis*, *A. gemina* and *A. ostoyae* were named the *A. ostoyae* cluster. A fourth group, the *A. gallica* cluster, included *A. calvescens*, *A. cepistipes*, *A. gallica*, *A. sinapina*, *A. nabsnona* and NABS X. Anderson and Stasovski (1992) concluded that three major groups of *Armillaria* species could be separated with respect to IGS-1 region sequences: (i) *A. borealis*, *A. gemina* and *A. ostoyae*, (ii) *A. calvescens*, *A. cepistipes*, *A. gallica*, *A. sinapina*, *A. nabsnona* and NABS X, and (iii) *A. mellea* and *A. tabescens*. Recently, Piercey-Normore et al. (1998) obtained anonymous nucleotide sequences (i.e. ramdom amplified polymorphic DNA products) from eight NABS of *Armillaria*. They proposed a phylogeny of *Armillaria* species that is very similar to that proposed by Anderson and Stasovski (1992). Overall, our results from DNA sequences support previous results on the phylogenetic relationships among *Armillaria* species; however, each rDNA region (i.e. IGS-1, ITS + 5.8S, and nLSU) provided different levels of phylogenetic signal. Sequence data from the IGS-1 region in this study can separate *A. nabsnona* and NABS X from the rest of Korhonen's (1995) group 4 – *A. gallica* cluster and Anderson and Stasovski's (1992) group 2. Of particular note is that *A. calvescens*, *A. sinapina*, *A. gallica* and *A. cepistipes* could not be separated on the basis of IGS-1 or ITS + 5.8S sequences. Thus, it appears that these four species cannot be identified using any techniques based on the IGS-1, ITS + 5.8S, or D-domain/3′ ends of nLSU.

   Although basidioma morphology can be used to reliably separate *A. mellea* and *A. tabescens*, the evolutionary relationship between these two species is not well resolved (Chillali et al. 1998). The nLSU was the only rDNA region that allowed unambiguous alignment of *A. mellea* and *A. tabescens* sequences with sequences of the other eight North
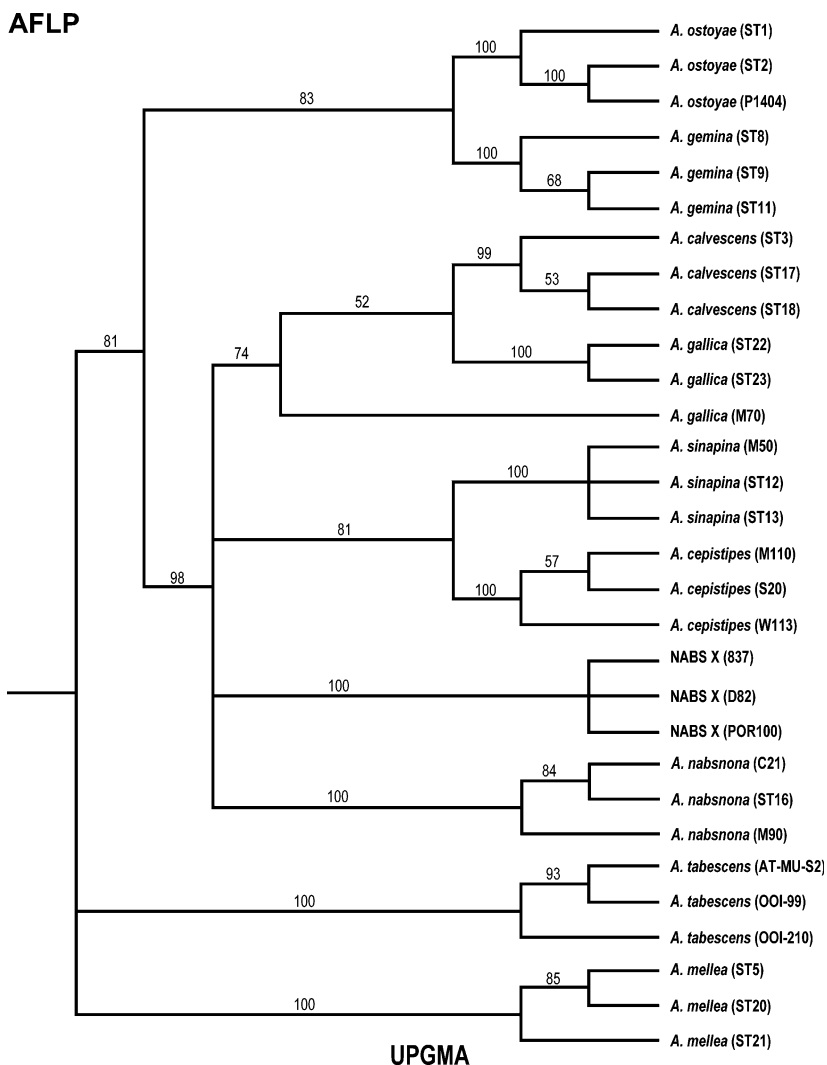
Fig. 5. UPGMA cluster of *Armillaria* species and genets generated from amplified fragment length polymorphism (AFLP) data. Bootstrap supports are indicated above branches based on 1000 bootstrap replicates

American *Armillaria* species. When *Pleurotus* sp. and *Xerula* sp. were used as outgroups for preliminary analysis of nLSU sequences, *A. mellea* was placed basal to the other of nine North American species, and *A. tabescens* was placed basal to the other eight species (unpublished data). However, more isolates from diverse geographical regions should be examined to confirm the phylogenetic relationship of *A. mellea* and *A tabescens. Armillaria mellea* was previously considered the most divergent of the North American species (BÉRUBÉ and DESSUREAULT 1988; ANDERSON et al. 1989; SMITH and ANDERSON 1989; ANDERSON and STASOVSKI 1992). Phylogenetic analysis of IGS-1 and ITS regions showed a distinct split between isolates from eastern and western North America, although the

definitive relationships among the geographical lineages remain undetermined (COETZEE et al. 2000). The previous analysis of *A. mellea* also showed well-supported clades among Europe, Asia and North America (COETZEE et al. 2000). In our study, three isolates of *A. mellea* derived from eastern/mid-western North America (Table 1) also showed divergent nLSU sequences (Fig. 4).

It has been proposed that the *A. ostoyae–A. gemina* group is closely related, but divergence has occurred (ANDERSON and STASOVSKI 1992; PIERCEY-NORMORE et al. 1998). PIERCEY-NORMORE et al. (1998) indicated the position of *A. gemina* as ancestral to *A. ostoyae*, but other studies have suggested that *A. gemina* was sympatrically derived from *A. ostoyae* (ANDERSON et al. 1989; ANDERSON and STASOVSKI 1992). Previous studies also showed similar nuclear DNA content for *A. ostoyae* and *A. gemina* (KIM et al. 2000). Our results confirmed previous studies that these two species are very closely related, but the phylogenetic relationships between them remain unresolved (Figs 2–4). Based on IGS-1 sequence analyses, a heterogeneous combination of sequence types was present in one *A. gemina* isolate (ST9) (Fig. 2): one sequence type (ST9-A) from the *A. gemina* isolate ST9 clustered with *A. gemina* genets (ST8-A, ST8-B and ST11) and the other sequence type (ST9-B) was clustered with *A. ostoyae* genets (ST1, ST2-A, ST2-B and P1404). This possible hybridization between *A. ostoyae* and *A. gemina* is strongly supported by both phylogenetic analyses (posterior probability 100%, bootstrap support 100%) (Fig. 2). However, a hybridization signature is not readily apparent in other rDNA regions because differences are insufficient to allow meaningful comparisons between *A. gemina* and *A. ostoyae*. All *A. ostoyae* isolates had 12 AFLP markers that were not shared by *A. gemina* isolates ST8 and ST11, and *A. gemina* isolates ST8 and ST11 had 18 AFLP markers that were not shared by any of the *A. ostoyae* isolates. Of these AFLP markers, *A. gemina* isolate ST9 shared one *A. ostoyae* marker and 16 *A. gemina* markers. These results may reflect a common ancestry of *A. ostoyae* and *A. gemina*. A polymorphism may have originated in the IGS region of *A. gemina*, and evolutionary processes may have rendered this region homogeneous in some lineages and heterogeneous in other lineages. Alternatively, *A. gemina* isolate ST9 may be derived from a past hybridization event, followed by introgression with *A. gemina*.

The close phylogenetic relationships hypothesized by ANDERSON and STASOVSKI (1992) and PIERCEY-NORMORE et al. (1998) among *A. calvescens*, *A. sinapina*, *A. gallica*, *A. nabsnona*, NABS X and *A. cepistipes* were supported by this study. However, we found support for a more distinct separation of *A. nabsnona* from these species based on sequence data from all three rDNA regions (Figs 2–4). KIM et al. (2000) reported that the nuclear content of *A. nabsnona* (0.133 pg per nucleus) is significantly smaller than *A. calvescens* (0.153 pg per nucleus), *A. sinapina* (0.150 pg per nucleus), *A. gallica* (0.168 pg per nucleus), NABS X (0.152 pg per nucleus), and *A. cepistipes* (0.152 pg per nucleus), while it is larger than *A. ostoyae* (0.113 pg per nucleus), *A. gemina* (0.120 pg per nucleus), and *A. mellea* (0.122 pg per nucleus). Of additional interest is that *A. gallica* (0.168 pg per nucleus) was previously shown to have a nuclear DNA content that was significantly larger than other North American *Armillaria* species (KIM et al. 2000), thereby providing evidence that *A. gallica* is a very distinct species in spite of any genetic relationships to other species.

One striking example of interspecific relationships is evident in isolates of NABS X, which contained heterogeneous sequence types within the ITS + 5.8S (Fig. 3). For each individual of NABS X tested, one sequence type (837-A, D82-A and POR-100A) clustered with *A. nabsnona*, while the other type (837-B, D82-B and POR-100B) remained separate from *A. nabsnona* (Fig. 3). However, IGS-1 sequence data showed that NABS X grouped with *A. calvescens*, *A. sinapina*, *A. gallica* and *A. cepistipes* (Fig. 2).

Of particular interest is that NABS X (0.152 pg per nucleus) has an intermediate nuclear DNA content between *A. gallica* (0.168 pg per nucleus) and *A. nabsnona* (0.133 pg per nucleus) (KIM et al. 2000). All *A. gallica* isolates had 11 AFLP markers that were not shared by any *A. nabsnona* isolates, and all *A. nabsnona* isolates had 16 AFLP markers that were not shared by any *A. gallica* isolates. Of these AFLP markers, NABS X shared 55% (six of 11) *A. gallica* markers and 25% (four of 16) *A. nabsnona* markers. The combined evidence from ITS + 5.8S sequences, IGS-1 sequences and AFLP markers suggests that NABS X may have originated by hybridization, although apparent phylogenetic implications differ according to rDNA regions. It is of additional interest to note that *A. calvescens* (0.153 pg per nucleus), *A. sinapina* (0.150 pg per nucleus) and *A. cepistipes* (0.152 pg per nucleus) also have intermediate nuclear DNA content (KIM et al. 2000). Of markers that distinguish *A. gallica* from *A. nabsnona*, *A. calvescens* shared 46% (five of 11) *A. gallica* markers and 6% (one of 16) *A. nabsnona* markers; *A. sinapina* shared 27% *A. gallica* markers and 6% *A. nabsnona* markers; and *A. cepistipes* shared 73% *A. gallica* markers and 25% *A. nabsnona* markers. More studies are needed to determine the potential role of hybridization in the creation and evolution of *Armillaria* species. It has been previously hypothesized that narrowly distributed *A. nabsnona* and NABS X are derived from more widely distributed *A. sinapina* (PIERCEY-NORMORE et al. 1998); however, this hypothesis is not supported by our present DNA and AFLP data. Hybridization events among *A. calvescens*, *A. sinapina*, *A. gallica*, *A. nabsnona*, NABS X and *A. cepistipes* are difficult to confirm because (i) these species have common ancestry, (ii) the species are genetically closely related, (iii) hybridization events may have occurred in the distant past and derived organisms have likely undergone selection pressure and introgression, and/or (iv) the hybridization events almost certainly involved isolates that were not included in this study.

Analyses of genetic relationships is further complicated by potential hybridization and gene flow among closely related *Armillaria* species. A previous study used a combination of molecular tools (PCR-RFLP and flow cytometry) to confirm interspecific mating in culture among biological species of *Armillaria* (*A. cepistipes*, *A. sinapina* and NABS X) (KIM et al. 2001). Heterogeneous rDNA sequences from individual genets also suggest that hybridization may occur in nature. Thus, limited gene flow may occur among closely related *Armillaria* species, and thereby cloud resolution of phylogenetic and genetic relationships. Hybridization between pathogens is currently a major concern due to the potential for hybrid individuals to possess new pathogenic traits (BRASIER 2000); however, more studies are needed to fully understand the impacts of hybridization among *Armillaria* species.

Repetitive DNA sequences like rDNA gene families are known to represent the result of concerted evolution (ELDER and TURNER 1995). However, our study showed that intraspecific homogenization of rDNA sequences is not the rule among the *Armillaria* species that we tested. Heterogeneous PCR products indicating intraspecific and intragenomic variations were common in all regions analysed, with the exception of the 5.8S rDNA. Direct PCR has been shown to detect 90% of the heterogeneous rDNA products in an individual and the relative peak height seems to reflect relative concentrations (RAUSCHER et al. 2002). This heterogeneity of IGS and ITS2 regions within single genomes or among strains has been reported in several groups of fungi (MARTIN 1990; O'DONNELL 1992; APPEL and GORDON 1996; O'DONNELL and CIGELNIK 1997; KAUSERUD and SCHUMACHER 2003). Currently, studies of fungal evolution are beginning to focus on protein-coding genes, such as elongation factor 1-α, β-tubulin, ribosomal polymerase B, mitochrondrial ATPase 6, mating-type genes, and chitin synthase genes, due to ease of alignment (BRUNS and SHEFFERSON 2004) and lack of heterogeneity. However, these single-copy genes are not as conserved as rDNA genes because selection is on the translated gene product, rather than the gene itself (BRUNS and SHEFFERSON 2004). BRUNS and SHEFFERSON (2004) suggested that structural ribosomal genes, such as nLSU rDNA, can be very useful for identification at high taxonomic levels due to a huge and growing

data set and protein-coding genes, such as mating type and synthase genes, hold great promise at lower taxonomic levels within fungi.

Sequences and variation of rDNA regions potentially contain very powerful phylogenetic information, which is typically unreported or underutilized. For precise phylogenetic analysis, it is essential that DNA sequence information is determined as accurately as possible. In this study, we maximized the phylogenetic signal from rDNA sequences through vigilant sequence editing, techniques to decipher heterogeneity within individuals, and elimination of ambiguity within data sets (Hanna 2005).

Compared with rDNA sequence information, AFLP marker data provided generally strong support for intraspecies grouping of *Armillaria* species. Genetic analyses of AFLP data from our selected isolates support the currently defined *Armillaria* species, based largely on the biological species concept. In addition, our data suggest that NABS X should be considered for elevation to formal species status. AFLP data can be used to discriminate among very closely related *Armillaria* species (i.e. *A. calvescens*, *A. sinapina*, *A. gallica* and *A. cepistipes*) that were difficult to identify using IGS-based PCR-RFLP methods (Harrington and Wingfield 1995; Kim et al. 2000). The increased resolution of AFLP-based analyses is expected because AFLP markers allow for simultaneous screening of multiple loci within the entire genome and reflect multiple sites within the genome that might be associated with phenotypes, pathogenicity, environmental adaptation, etc. In contrast, sequencing of one or more regions within the genome can provide higher order phylogenies of populations by comparing the evolutionary rates of individual genotypes.

The results from this study suggest that AFLP data can be used to discriminate among 10 NABS of *Armillaria*. Several AFLP genetic markers offer potential for distinguishing currently available NABS of *Armillaria* in future biological, ecological and taxonomic studies. Unique loci associated with each North American *Armillaria* species could be sequenced and used to develop primers for identifying biological species. However, more AFLP markers are needed to develop species-specific probes for discriminating between *A. calvescens* and *A. gallica* because of their close genetic relationship. To our knowledge, this is the first study to apply AFLP data for analysis of North American *Armillaria* species in comparison with data inferred from rDNA sequences.

An overriding goal of this study was to further characterize a set of diploid tester strains (Kim et al. 2000) for use in future studies to help characterize additional isolates of *Armillaria*. Our study provides the foundation to examine further questions about intraspecific variation, global variation, interspecific relationships, intraspecific relationships, etc. Such efforts should contribute to a better understanding of biological, ecological and taxonomic relationships among *Armillaria* species.

Acknowledgements

Résumé

Caractérisation des espèces d'Armillaria d'Amérique du Nord: relations génétiques déterminées à partir des séquences de l'ADN ribosomal et de marqueurs AFLP

Les relations phylogénétiques et génétiques entre 10 espèces nord-américaines d'*Armillaria* ont été analysées à partir d'une part de données de séquences de l'ADN ribosomal (ADNr), incluant l'espaceur inter-génique (IGS-1), les espaceurs internes transcrits et le 5.8S associé (ITS + 5.8S), et

l'ADNr nucléaire de la grande sous-unité (nLSU), d'autre part de marqueurs AFLP. D'après les données de séquence de l'ADNr, la région nLSU est moins variable entre espèces d'*Armillaria* que les régions ITS + 5.8S et IGS-1 (nLSU < ITS + 5.8S < IGS-1). Les analyses phylogénétiques basées sur les séquences de l'ADNr suggèrent que *A. mellea*, *A. tabescens*, et *A. nabsnona* sont bien séparées des autres espèces d'*Armillaria* (*A. ostoyae*, *A. gemina*, *A. calvescens*, *A. sinapina*, *A. gallica*, NABS X, et *A. cepistipes*). Plusieurs espèces d'*Armillaria* (*A. calvescens*, *A. sinapina*, *A. gallica*, NABS X, et *A. cepistipes*) se trouvent regroupées avec l'utilisation de ces séquences. En se basant sur les isolats utilisés dans cette étude, il apparaît donc que les techniques basées sur l'IGS-1, ITS + 5.8S, et/ou le domaine D/extrémité 3′ de nLSU sont insuffisantes pour distinguer *A. calvescens*, *A. sinapina*, *A. gallica*, et *A. cepistipes*. Toutefois, les données d'AFLP ont permis de différencier ces espèces et l'analyse AFLP est cohérente avec la classification taxonomique établie à partir des méthodes conventionnelles (morphologie et tests d'interfertilité). Nos résultats montrent l'intérêt des marqueurs AFLP pour distinguer les différentes espèces biologiques nord-américaines d'*Armilllaria* pour des études de biologie, d'écologie et de taxonomie.

Zusammenfassung

Charakterisierung von nordamerikanischen Armillaria-Arten: Bestimmung der genetischen Beziehungen anhand von ribosomalen rDNA-Sequenzen und AFLP-Markern

Die phylogenetischen und genetischen Beziehungen zwischen zehn nordamerikanischen *Armillaria*-Arten wurden mit Hilfe von Sequenzdaten der ribosomalen DNA (rDNA), welche die Regionen IGS-1, ITS + 5,8 S und nLSU einschliesst, sowie mit AFLP-Markern analysiert. Die Sequenzdaten ergaben, dass die nLSU-Region bei *Armillaria*-Arten weniger stark variiert als die ITS + 5,8S sowie die IGS-1 Regionen (nLSU < ITS + 5,8 S <IGS-1). Aus den phylogenetischen Analysen der rDNA-Sequenzen ergab sich eine klare Trennung von *A. mellea*, *A. tabescens* und *A. nabsnona* von den übrigen Arten (*A. ostoyae*, *A. gemina*, *A. calvescens*, *A. sinapina*, *A. gallica*, NABS X und *A. cepistipes*). Verschiedene Arten (*A. calvescens*, *A. sinapina*, *A. gallica*, NABS X und *A. cepistipes*) bildeten aufgrund ihrer rDNA-Sequenzen eine Gruppe. Am untersuchten Material zeigte sich, dass Techniken, die auf IGS1, ITS + 5,8 S und/oder D-Domäne/3′-Enden der nLSU basieren, nicht zuverlässig zwischen *A. calvescens*, *A. sinapina*, *A. gallica* und *A. cepistipes* unterscheiden können. Im Gegensatz dazu konnten diese Arten mit AFLP-Markern voneinander abgegrenzt werden und die Analyse der AFLP-Daten bestätigte die taxonomische Klassifikation aufgrund konventioneller Methoden (Morphologie und Interfertilitätstests). Die Befunde zeigen, dass AFLP-Marker zur Unterscheidung der derzeit akzeptierten nordamerikanischen biologischen Arten von *Armillaria* sowie für zukünftige biologische, ökologische und taxonomische Untersuchungen eingesetzt werden können.

References

ANDERSON, J. B.; KOHN, L. M., 1995: Clonality in soilborne, plant-pathogenic fungi. Annu. Rev. Phytopathol. **33**, 369–391.

ANDERSON, J. B.; STASOVSKI, E., 1992: Molecular phylogeny of Northern Hemisphere species of *Armillaria*. Mycologia **84**, 506–516.

ANDERSON, J. B.; ULLRICH, R. C., 1979: Biological species of *Armillaria mellea* in North America. Mycologia **71**, 402–414.

ANDERSON, J. B.; PETSCHE, D. M.; SMITH, M. L., 1987: Restriction fragment polymorphisms in biological species of *Armillaria mellea*. Mycologia **79**, 69–76.

ANDERSON, J. B.; BAILEY, S. S.; PUKKILA, P. J., 1989: Variation in ribosomal DNA of *Armillaria*, a genus of root-infecting fungi. Evolution **43**, 1652–1662.

APPEL, D. J.; GORDON, T. R., 1996: Relationships among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. Mol. Plant Microbe Interact. **9**, 125–138.

BANIK, M. T.; VOLK, T. J.; BURDSALL, H. H., Jr, 1996: *Armillaria* species of the Olympic Peninsula of Washington state, including confirmation of North American biological species XI. Mycologia **88**, 492–496.

BÉRUBÉ, J. A.; DESSUREAULT, M., 1988: Morphological characterization of *Armillaria sinapina* sp. nov. Can. J. Bot. **66**, 2027–2034.

BRASIER, C. M., 2000: The rise of hybrid fungi. Nature **405**, 134–135.

BRUNS, T. D.; SHEFFERSON, R. P., 2004: Evolutionary studies of ectomycorrhizal fungi: recent advances and future directions. Can. J. Bot. **82**, 1122–1132.

CHILLALI, M.; WIPF, D.; GUILLAUMIN, J.-J.; MOHAMMED, C.; BOTTON, B., 1998: Delineation of the European *Armillaria* species based on the sequences of the internal transcribed spacer (ITS) of ribosomal DNA. New Phytol. **138,** 553–561.

COETZEE, M. P. A.; WINGFIELD, B. D.; HARRINGTON, T. C.; DALEVI, D.; COUTINHO, T. A.; WINGFIELD, M. J., 2000: Geographic diversity of *Armillaria mellea* s.s. based on phylogenetic analysis. Mycologia **92,** 105–113.

COETZEE, M. P. A.; WINGFIELD, B. D.; BLOOMER, P.; RIDLEY, G. S.; KILE, G. A.; WINGFIELD, M. J., 2001: Phylogenetic relationships of Australian and New Zealand *Armillaria* species. Mycologia **93,** 887–896.

COETZEE, M. P. A.; WINGFIELD, B. D.; BLOOMER, P.; RIDLEY, G. S.; WINGFIELD, M. J., 2003: Molecular identification and phylogeny of *Armillaria* isolates from South America and Indo-Malaysia. Mycologia **95,** 285–293.

CREASE, T. J.; LYNCH, M., 1991: Ribosomal DNA variation in *Daphnia pulex*. Mol. Biol. Evol. **8,** 620–640.

DUCHESNE, L. C.; ANDERSON, J. B., 1990: Location and direction of transcription of the 5S rRNA gene in *Armillaria*. Mycol. Res. **94,** 266–269.

ELDER, J. F., Jr; TURNER, B. J., 1995: Concerted evolution of repetitive DNA sequences in eukaryotes. Q. Rev. Biol. **70,** 297–320.

ERIKSSON, T.; HIBBS, M. S.; YODER, A. D.; DELWICHE, C. F.; DONOGHUE, M. J., 2003: The phylogeny of Rosoideae (Rosaceae) based on sequences of the internal transcribed spacers (ITS) of the nuclear ribosomal DNA and the TRNL/F region of chloroplast DNA. Int. J. Plant Sci. **164,** 197–211.

FELL, J. W.; BOEKHOUT, T.; FONSECA, A.; SCORZETTI, G.; TALLMAN, A., 2000: Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. Int. J. Syst. Evol. Microbiol. **50,** 1351–1371.

FELSENSTEIN, J., 1985: Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39,** 783–791.

GARDES, M.; BRUNS, T. D., 1993: ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rust. Mol. Ecol. **2,** 113–118.

GEZAHGNE, A.; COETZEE, M. P. A.; WINGFIELD, B. D.; WINGFIELD, M. J.; ROUX, J., 2004: Identification of the Armillaria root rot pathogen in Ethiopian plantations. For. Pathol. **34,** 133–145.

HALL, T. A., 1999: BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids. Symp. Ser. **41,** 95–98.

HALL, G. H., 2001: Phylogenetic Trees Made Easy: a How-to Manual for Molecular Biologists. Sunderland, MA: Sinauer Associates, Inc., 164 pp.

HANNA, J. W., 2005: *Armillaria ostoyae*: genetic characterization and distribution in the western United States. MS Thesis, University of Idaho, Moscow, ID, 116 pp.

HARRINGTON, T. C.; WINGFIELD, B. D., 1995: A PCR-based identification method for species of *Armillaria*. Mycologia **87,** 280–288.

HASEGAWA, M.; KISHINO, H.; YANO, T., 1985: Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22,** 160–174.

HINTIKKA, V., 1973: A note on the polarity of *Armillariella mellea*. Karstenia **13,** 32–39.

HOPPLE, J. S., Jr; VILGALYS, R., 1999: Phylogenetic relationships in the mushroom genus *Coprinus* and dark-spored allies based on sequence data from the nuclear gene coding for the large ribosomal subunit RNA: divergent domains, outgroups and monophyly. Mol. Phylogenet. Evol. **13,** 1–19.

HUELSENBECK, J. P.; RONQUIST, F., 2001: MrBayes: Bayesian inference of phylogenetic trees. Biometrics **17,** 754–755.

JAHNKE, K. D.; BAHNWEG, G.; WORRALL, J. J., 1987: Species delimitation in the *Armillaria mellea* complex by analysis of nuclear and mitochondrial DNAs. Trans. Br. Mycol. Soc. **88,** 572–575.

KAUSERUD, H.; SCHUMACHER, T., 2003: Genetic structure of Fennoscandian populations of the threatened wood-decay fungus *Fomitopsis rosea*. Mycol. Res. **107,** 155–163.

KIM, M.-S.; KLOPFENSTEIN, N. B.; MCDONALD, G. I.; ARUMUGANATHAN, K.; VIDAVER, A. K., 2000: Characterization of North American *Armillaria* species by nuclear DNA content and RFLP analysis. Mycologia **92,** 874–883.

KIM, M.-S.; KLOPFENSTEIN, N. B.; MCDONALD, G. I.; ARUMUGANATHAN, K.; VIDAVER, A. K., 2001: Use of flow cytometry, fluorescence microscopy, and PCR-based techniques to assess intraspecific and interspecific matings of *Armillaria* species. Mycol. Res. **105,** 153–163.

KORHONEN, K., 1978: Infertility and clonal size in the *Armillaria* complex. Karstenia **18,** 31–42.

KORHONEN, K., 1995: *Armillaria* since Elias Fries. Symb. Bot. Upsal. **30,** 153–161.

KUZOFF, R. K.; SWEERE, J. A.; SOLTIS, D. E.; SOLTIS, P. S.; ZIMMER, E. A., 1998: The phylogenetic potential of entire 26S rDNA sequences in plants. Mol. Biol. Evol. **15**, 251–263.

MARTIN, F. N., 1990: Variation in the ribosomal DNA repeat unit within single-oospore isolates of the genus *Pythium*. Genome **33**, 585–591.

MICHOT, B.; HASSOUNA, N.; BACHELLERIE, J., 1984: Secondary structure of mouse 28S rRNA and a general model for the folding of the large rRNA in eukaryotes. Nucleic Acids Res. **12**, 4259–4279.

MICHOT, B.; QU, H.-L.; BACHELLERIE, J. P., 1990: Evolution of large-subunit ribosomal RNA structure: the diversification of divergent D3 domain among major phylogenetic groups. Eur. J. Biochem. **188**, 219–230.

MILLER, O. K.; JOHNSON, J. L.; BURDSALL, H. H.; FLYNN, T., 1994: Species delimitation in North American species of *Armillaria* as measured by DNA reassociation. Mycol. Res. **98**, 1005–1011.

MONCALVO, J.-M.; LUTZONI, F. M.; REHNER, S. A.; JOHNSON, J.; VILGALYS, R., 2000: Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. Syst. Biol. **49**, 278–305.

MONCALVO, J.-M.; VILGALYS, R.; REDHEAD, S. A.; JOHNSON, J. E.; JAMES, T. Y.; AIME, M. C.; HOFSTETTER, V.; VERDUIN, S. J. W.; LARSSON, E.; BARONI, T. J.; THORN, R. G.; JACOBSSON, S.; CLEMENCON, H.; MILLER, O. K., JR, 2002: One hundred and seventeen clades of euagarics. Mol. Phylogenet. Evol. **23**, 357–400.

MOTTA, J. J.; KORHONEN, K., 1986: A note on *Armillaria mellea* and *Armillaria bulbosa*. Mycologia **78**, 963–965.

NEI, M.; LI, W. H., 1979: Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. **76**, 5269–5273.

NYLANDER, J. A. A., 2003: MrModeltest 1.0b. A Simplified Version of David Posada's "Modeltest 3.06". Uppsala, Sweden: Department of Systematic Zoology, http://www.ebc.uu.se/systzoo/staff/nylander.html.

O'DONNELL, K., 1992: Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). Curr. Genet. **22**, 213–220.

O'DONNELL, K.; CIGELNIK, E., 1997: Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. **7**, 103–116.

OTIENO, W.; PÉREZ-SIERRA, A.; TERMORSHUIZEN, A., 2003: Characterization of *Armillaria* isolates from tea (*Camellia sinensis*) in Kenya. Mycologia **95**, 160–175.

PÉREZ-SIERRA, A.; GUILLAUMIN, J.-J.; SPOONER, B. M.; BRIDGE, P. D., 2004: Characterization of *Armillaria heimii* from Africa. Plant Pathol. **53**, 220–230.

PIERCEY-NORMORE, M. D.; EGGER, K. N.; BÉRUBÉ, J. A., 1998: Molecular phylogeny and evolutionary divergence of North American Biological Species of *Armillaria*. Mol. Phylogenet. Evol. **10**, 49–66.

RAUSCHER, J. T.; DOYLE, J. J.; BROWN, H. D., 2002: Internal transcribed spacer repeat-specific primers and the analysis of hybridization in the *Glycine tomentella* (Leguminosae) polyploid complex. Mol. Ecol. **11**, 2691–2702.

SAITOU, N.; NEI, M., 1987: The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**, 406–425.

SCHULZE, S.; BAHNWEG, G.; TESCHE, M.; SANDERMANN, H., JR, 1995: Identification of European *Armillaria* species by restriction-fragment-length polymorphisms of ribosomal DNA. Eur. J. For. Pathol. **25**, 214–223.

SCHULZE, S.; BAHNWEG, G.; MÖLLER, E. M.; SANDERMANN, H., JR, 1997: Identification of genus *Armillaria* by specific amplification of an rDNA-ITS fragment and evaluation of genetic variation within *A. ostoyae* by rDNA-RFLP and RAPD analysis. Eur. J. For. Pathol. **27**, 225–239.

SHAW, C. G., III; KILE, G. A., 1991: Armillaria Root Disease. USDA Forest Service, Agriculture Handbook No. 691, 233 pp.

SIMMONS, M. P.; OCHOTERENA, H., 2000: Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. **49**, 369–381.

SMITH, M. L.; ANDERSON, J. B., 1989: Restriction fragment length polymorphisms in mitochondrial DNA's of *Armillaria*: identification of North American biological species. Mycol. Res. **93**, 247–256.

SMITH, M. L.; DUCHESNE, L. C.; BRUHN, J. N.; ANDERSON, J. B., 1990: Mitochondrial genetics in a natural population of the plant pathogen *Armillaria*. Genetics **126**, 575–582.

SWOFFORD, D. L., 2001: PAUP*: Phylogenetic Analysis Using Parsimony and Other Methods (PAUP* version 4.0 beta 10). Sunderland, MA: Sinauer.

TERASHIMA, K.; CHA, J. Y.; YAJIMA, T.; IGARASHI, T.; MIURA, K., 1998: Phylogenetic analysis of Japanese *Armillaria* based on the intergenic spacer (IGS) sequences of their ribosomal DNA. Eur. J. For. Pathol. **28**, 11–19.

TERMORSHUIZEN, A. J.; ARNOLDS, E. J. M., 1987: On the nomenclature of the European species of *Armillaria mellea* group. Mycotaxon **30**, 101–116.

TERMORSHUIZEN, A. J.; ARNOLDS, E. J. M., 1997: Compatibility groups, species concepts and nomenclature in European *Armillaria* species. Mycotaxon **65**, 263–272.

VELDMAN, G. M.; KLOOTWIJK, J.; DE REGT, V. C. H. F.; RUDI, J., 1981: The primary and secondary structure of yeast 26S rRNA. Nucleic Acids Res. **9**, 6935–6952.

VOLK, T. J.; BURDSALL, H. H., JR, 1995: A Nomenclatural Study of *Armillaria* and *Armillariella* Species (Basidiomycotina, Tricholomataceae). Synopsis Fungorum 8, Oslo, Norway, 121 pp.

VOLK, T. J.; BURDSALL, H. H., JR; BANIK, M. T., 1996: *Armillaria nabsnona*, a new species from western North America. Mycologia **88**, 484–491.

VOS, P.; HOGERS, R.; BLEEKER, M.; REIJANS, M.; DE LEE, T.; HORNES, M.; FRIJTERS, A.; POT, J.; PELEMAN, J.; KUIPER, M.; ZABEAU, M., 1995: AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. **23**, 4407–4414.

WEISS, M.; OBERWINKLER, F., 2001: Phylogenetic relationships in *Auriculariales* and related groups – hypotheses derived from nuclear ribosomal DNA sequences. Mycol. Res. **105**, 403–415.

WHITE, T. J.; BRUNS, T. D.; LEE, S. B.; TAYLOR, J. W., 1990: Analysis of phylogenic relationships by amplification and direct sequencing of ribosomal DNA genes. In: PCR Protocols: a Guide to Methods and Applications. Ed. by INNIS, M. A.; GELFAND, D. H.; SNINSKY, J. J.; WHITE, T. J. New York: Academic Press, pp. 315–322.

WHITE, E. E.; DUBETZ, C. P.; CRUICKSHANK, M. G.; MORRISON, D. J., 1998: DNA diagnostic for *Armillaria* species in British Columbia: within and between species variation in the IGS-1 and IGS-2 regions. Mycologia **90**, 125–131.

YANG, Z., 1993: Maximum likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. Mol. Biol. Evol. **10**, 1396–1402.